In Vitro Assessment of IL-4- or IL-13-Mediated Changes in the Structural Components of Keratinocytes in Mice and Humans

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T helper type 2 (Th2) cytokines, IL-4 and IL-13, attenuate the expression of genes that regulate epidermal cellular structures and the barrier function at the terminal stage of keratinocyte differentiation. However, whether these Th2 cytokines act at earlier stages remains unknown. We investigated the roles of cytokines in expression levels of mRNAs and/or proteins in primary mouse keratinocytes and human keratinocyte HaCaT cells at earlier stages. We showed that IL-4 downregulated the expression levels of *Krt1*, *Krt10*, *Dsg1*, and *Dsc1* via IL-4Rα- and signal transducer and activator of transcription factor 6 (STAT6)-dependent mechanisms in differentiating mouse keratinocytes at early stages. As the expression levels of keratin-1 and -10 in the keratinocytes transiently expressing an active form of STAT6 were not downregulated, STAT6 and other IL-4-induced molecules may synergistically regulate this expression. The restoration of the downregulated expression levels of *Krt1* and *Krt10* induced by IL-4 with the MEK (mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase kinase) inhibitor U0126 indicated the involvement of the p44/42 MAPK signaling pathway in the attenuated expression. IL-13 also downregulated the expression of the four genes. Furthermore, IL-4 or IL-13 caused the downregulation of these genes in HaCaT cells and promoted the fragmentation of cell sheets with mechanical stress. Our results showed that IL-4 or IL-13 acted on differentiating keratinocytes *in vitro* at early stages to attenuate the gene expression.

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INTRODUCTION

T helper type 2 (Th2)-type immune responses are involved in the development of atopic dermatitis (AD) and other allergic diseases (Aleksza *et al.*, 2002; La Grutta *et al.*, 2005). The Th2 cytokine IL-4 and epithelial cytokines, including thymic stromal lymphopoietin, IL-25, and IL-33, have a role in the initiation and/or amplification of Th2-type immune responses via Th2 and nonlymphoid cells (Saenz *et al.*, 2008; Paul and Zhu, 2010; Saenz *et al.*, 2010). Along with IL-4, the Th2 cytokines IL-5 and IL-13 are expressed in the lesional skin of AD patients at significantly higher levels than in the skin of nonatopic individuals (Hamid *et al.*, 1994; Jeong *et al.*, 2003; Nomura *et al.*, 2003; Neis *et al.*, 2006). Mice overexpressing these cytokines develop spontaneous AD and asthma (Lee and Flavell, 2004), whereas the skin-specific overexpression of IL-4 in mice induces the spontaneous development of AD (Chan *et al.*, 2001). Therefore, high expression levels of these Th2 cytokines have a role in the abnormal development of the skin.

The main cellular component of the epidermis in the skin is keratinocytes, which undergo differentiation from the basal layer toward the spinous layer and granular layer to the stratum corneum as they migrate. Keratins are the structural proteins of keratinocytes and form the intermediate filament cytoskeleton in the epidermis (Candi *et al.*, 2005). Proliferating keratinocytes in the basal layer express keratin (KRT) 5 and KRT14, whereas those in the spinous/granular layers express KRT1 and KRT10 (Candi *et al.*, 2005; Simpson *et al.*, 2011). Desmosomal cadherins, i.e., desmogleins (DSGs) and desmocollins (DSCs), link the intracellular network of KRT intermediate filaments and connect neighboring keratinocytes (Candi *et al.*, 2005; Simpson *et al.*, 2011). DSG3 and DSC3 are expressed at the highest level in the basal layer and at the lowest level in the granular layer, whereas DSG1 is expressed at the lowest level in the basal layer and at the highest level in the granular layer and DSC1 is expressed at the lowest level in the spinous layer and at the highest level in the granular layer (Simpson *et al.*, 2011).

After the induction of Th2 cytokines in patients with AD, keratinocytes modify structural components and/or the

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Abbreviations: AD, atopic dermatitis; DSC, desmocollin; DSG, desmoglein; KRT, keratin; MAPK, mitogen-activated protein kinase; Th2, T helper type 2

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barrier function of the stratum corneum. Attenuated expression levels of various components, i.e., involucrin, filaggrin, and loricrin, are detected in AD skin with abundant IL-13 (Suarez-Farinas *et al.*, 2011) and IL-4- and/or IL-13-treated human keratinocytes (Howell *et al.*, 2007; Kim *et al.*, 2008). Even a low level of IL-4 attenuates the expression levels of *Flg*, *Ivl*, and *Lor* in the mouse epidermis (Sehra *et al.*, 2010).

Most studies of the roles of Th2 cytokines in structural components and/or the functions of keratinocytes have focused on the terminal stage of keratinocyte differentiation in the stratum corneum (Howell *et al.*, 2007; Kim *et al.*, 2008; Sehra *et al.*, 2010; Suarez-Farinas *et al.*, 2011). If modified structures of keratinocytes are present at earlier stages of differentiation, they may influence subsequent differentiation events and/or the stability of epidermal integrity. Therefore, events occurring in layers other than the stratum corneum in a Th2 cytokine–dominant allergic environment require close scrutiny. We herein investigated the roles of Th2 cytokines *in vitro* in primary mouse keratinocytes and human keratinocyte HaCaT cells at earlier stages of differentiation. Our study showed that IL-4 or IL-13 attenuated the expression levels of genes at early stages of differentiation in mouse keratinocytes and human keratinocyte HaCaT cells and disrupted the integrity of cell sheets of human keratinocyte HaCaT cells. These findings demonstrate that IL-4 or IL-13 modifies the structural components of keratinocytes at both the terminal and earlier stages of differentiation *in vitro*. The detailed interplay between macromolecules during the exposure of keratinocytes to IL-4 or IL-13 that results in the loss of integrity of the cell architecture remains to be investigated.

RESULTS

IL-4 attenuates the expression of genes encoding structural components of primary mouse keratinocytes at early stages

To evaluate the effects of IL-4 on the expression of genes encoding structural components of differentiating keratinocytes in the basal layer through the outer layer of the epidermis, a series of real-time reverse-transcriptase-PCR experiments were performed to examine KRT genes, i.e., *Krt5*, *Krt14*, *Krt1*, and *Krt10* (Figure 1a), and desmosomal cadherin genes, i.e., *Dsg3*, *Dsc3*, *Dsg1*, and *Dsc1* (Figure 1b). The mRNA expression profiles for KRTs in the keratinocytes indicated that IL-4 attenuated the *Krt1* and *Krt10* expression levels and slightly downregulated the *Krt5* and *Krt14* expression levels (Figure 1a). The protein profiles were then examined for genes that were attenuated in their mRNA expression levels in the presence of IL-4. The protein expression levels of KRT1 and KRT10 exhibited the identical pattern, whereas the expression levels of KRT5 and KRT14 were not statistically significant (Figure 1c and d). The mRNA expression levels of *Dsg1a* and *Dsc1* were attenuated in the keratinocytes stimulated with IL-4, whereas the expression levels of *Dsg3* and *Dsc3* did not significantly change (Figure 1b). In contrast, the pattern of the protein expression levels of DSG1 and DSC1 did not show any changes in the differentiating keratinocytes with and without IL-4 (Figure 1e and f). Therefore, the present study showed that IL-4 downregulated the expression levels of both mRNA and proteins of *Krt1* and *Krt10* and the expression levels of mRNA only of *Krt5*, *Krt14*, *Dsg1a*, and *Dsc1*.

IL-13, but not IL-5, downregulates the mRNA expression levels of *Krt1*, *Krt10*, *Dsg1a*, and *Dsc1* in mouse keratinocytes

In addition to IL-4, IL-5 and IL-13 are strongly associated with the pathogenesis of AD (Jeong *et al.*, 2003); however, the effects of these cytokines on the expression of structural components of keratinocytes remain unknown. We investigated whether IL-5 or IL-13 modifies the expression of genes encoding structural components of differentiating keratinocytes at early stages. Compared with the expression of keratinocytes without cytokines, the mRNA expression levels of *Krt1* and *Krt10* were downregulated by IL-13 to the same extent or more than that downregulated by IL-4 (Figure 2a). In addition, IL-13 strongly attenuated the expression of *Dsc1* mRNA but only slightly attenuated the expression of *Dsgla* mRNA (Figure 2b). In contrast, IL-5 did not affect the mRNA expression of KRTs or desmosomal components in the present study (Figure 2a and b). As IL-4 and IL-13 each signal through the IL-4 receptor-α (IL-4Rα) chain (Wills-Karp and Finkelman, 2008), we examined whether the IL-4Rα chain is required for the regulation of KRT and desmosomal cadherin genes induced by IL-4 or IL-13. No reductions in the mRNA expression of KRTs (Figure 2c) or desmosomal components (Figure 2d) were detected in *Il4ra*-deficient keratinocytes, suggesting that IL-4 and IL-13 both attenuate the expression levels of *Krt1*, *Krt10*, *Dsg1a*, and *Dsc1* mRNAs via the IL-4Rα chain.

The STAT6 signaling pathway is essential, but likely insufficient, for IL-4- or IL-13-mediated changes in the gene expression of mouse differentiating keratinocytes

Both IL-4 and IL-13 activate STAT6 in human keratinocytes (David *et al.*, 2001; Bao *et al.*, 2012), and attenuated mRNA expression levels of *Ivl* and *Lor* have been reported in skin biopsies of *Stat6*-transgenic mice (Kim *et al.*, 2008). To evaluate whether the STAT6 signaling pathway is involved in the IL-4- or IL-13-mediated attenuation of the mRNA expression of structural components, we analyzed the expression of KRTs and desmosomal components in differentiating keratinocytes derived from *Stat6*-deficient mice by using real-time reverse-transcriptase-PCR. STAT6 was phosphorylated after stimulation with either IL-4 or IL-13 at a peak of 10 minutes after adding either one of the cytokines to differentiating keratinocytes (Figure 3a). In the presence of IL-4 or IL-13, differentiating keratinocytes derived from *Stat6*-deficient mice did not show attenuated mRNA expression levels of the KRT genes *Krt1* and *Krt10* (Figure 3b) or the desmosomal component genes *Dsg1a* and *Dsc1* (Figure 3c). Therefore, the STAT6 signaling pathway as a target of the IL-4 receptor is essential for IL-4 or IL-13 to attenuate the mRNA expression levels of *Krt1*, *Krt10*, *Dsg1a*, and *Dsc1*.

We then investigated whether STAT6 activation is sufficient for the attenuated expression of these structural proteins.

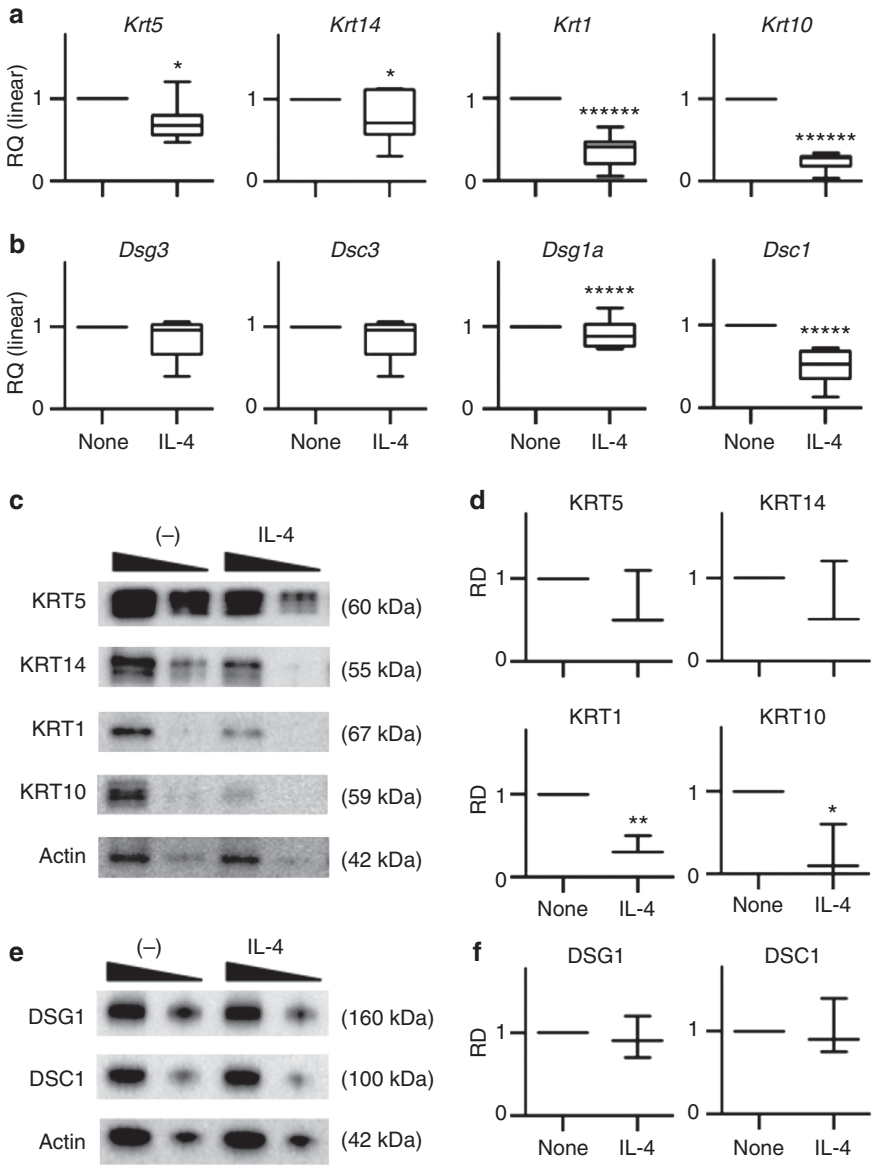

Figure 1. IL-4 attenuates the expression levels of *Krt1*, *Krt10*, *Dsg1a*, and *Dsc1* in differentiating primary mouse keratinocytes. The keratinocytes were differentiated for 9 days. IL-4 was added for the last 2 days of the culture. A real-time PCR analysis of (**a**) *Krt1*, *Krt5*, *Krt10*, *Krt14*, (**b**) *Dsg1a*, *Dsg3*, *Dsc1*, and *Dsc3* with or without IL-4 stimulation. The data were normalized to the *Ppia* mRNA expression using the comparative *CT* method. The relative quantity (RQ) of genes is depicted as the mRNA expression in the differentiating keratinocytes without any cytokines ($n=7$). Asterisk, statistically significant (a matched-pair analysis). A western blotting analysis of (**c**) keratin 1 (KRT1), KRT5, KRT10, KRT14, and actin ($n=3$) and (**e**) desmoglein 1 (DSG1) and desmocollin 1 (DSC1; $n=3$). The amount of soluble fraction of each lysate in the right lane is one-third of that presented in the left lane. (**d**, **f**) Densitometry plots of **c** and **e**. RD, relative density; asterisk, statistically significant (a matched-pair analysis).

Using the keratinocytes transfected with an empty vector or the active form of STAT6 (STAT6VT) in an expression vector, the expression levels of KRT1 and KRT10 were analyzed by gating transfected cells on flow cytometry. No significant differences were observed between the empty vector–transfected cells and the STAT6VT vector–transfected cells for either KRT1 or KRT10 at the mean fluorescence intensity, suggesting that the activation of STAT6 by itself is not sufficient to attenuate the KRT1 and KRT10 expression levels (Figure 3d).

The p44/42 mitogen-activated protein kinase (MAPK) signaling pathway is indispensable for IL-4-mediated changes in the mRNA expression levels of *Krt1* and *Krt10*, but not IL-4-mediated changes in the mRNA expression of *Dsc1*, in mouse keratinocytes

MAPKs are generally involved in proliferation, survival, differentiation, and cell death (Seger and Krebs, 1995; Eckert *et al.*, 2002). The p44/42 MAPK signaling pathway is activated in response to IL-4 or IL-13 in human keratinocyte HaCaT cells (David *et al.*, 2001). In this study, we examined the

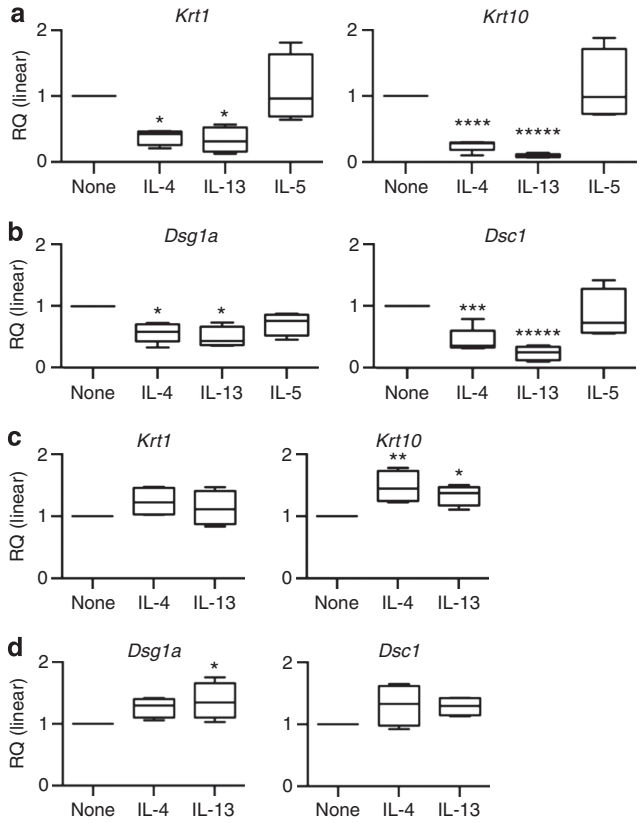

Figure 2. Similar to IL-4, IL-13 downregulates the expression of genes encoding structural components of primary mouse keratinocytes via IL-4 receptor alpha. Keratinocytes obtained from wild-type mice (**a**, **b**) and *Il4ra*-deficient mice (**c**, **d**) were differentiated for 9 days. IL-4, IL-13, or IL-5 was added for the last 2 days of the culture. A real-time PCR analysis of (**a**, **c**) *Krt1*, *Krt10*, (**b**, **d**) *Dsg1a*, and *Dsc1* with or without cytokine stimulation ($n=4$). Refer to Figure 1 for the normalization of the data and the relative quantities (RQs) of the genes. Asterisk, statistically significant (one-way analysis of variance with Dunnett's *post hoc* test).

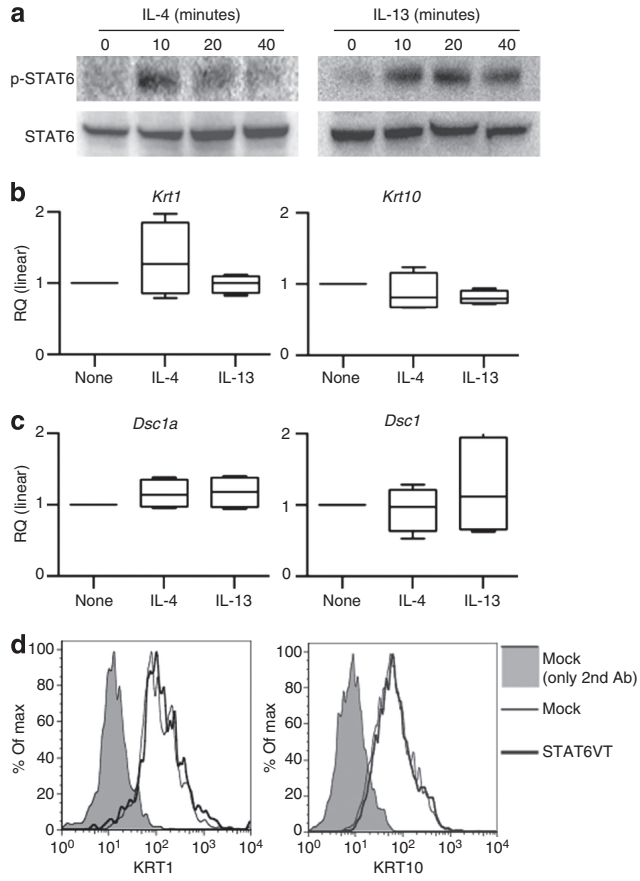

Figure 3. The signal transducer and activator of transcription factor 6 (STAT6) signaling pathway is essential for the IL-4-mediated attenuation of the expression of genes encoding structural components of primary mouse keratinocytes. (**a**) A western blotting analysis of phospho-STAT6 or total STAT6 ($n=3$). The keratinocytes were differentiated for 6 days. After overnight starvation, the cells were stimulated with IL-4 or IL-13 for 10, 20, or 40 minutes. (**b**, **c**) A real-time PCR analysis of (**b**) *Krt1*, *Krt10*, (**c**) *Dsg1a*, and *Dsc1* with or without IL-4 or IL-13 stimulation ($n=4$). *Stat6*-deficient keratinocytes were differentiated for 9 days. IL-4 or IL-13 was added for the last 2 days of the culture. Refer to Figure 1 for the normalization of the data and the relative quantities (RQs) of the genes. One-way analysis of variance with Dunnett's *post hoc* test indicated no differences between the cells treated with or without IL-4 or IL-13. (**d**) Intracellular staining of keratin 1 (KRT1) and KRT10 ($n=3$). Only with the secondary antibody was used as a control. The expression in $EGFP^+$-transfected cells is depicted.

response(s) of p44/42 MAPK to IL-4 or IL-13 in mouse differentiating keratinocytes. p44/42 MAPK was phosphorylated by IL-4 or IL-13 at a peak of 5 minutes after stimulation (Figure 4a). As a previous report has shown in human keratinocytes (David *et al.*, 2001), the phosphorylation of p42 MAPK was stronger than that of p44 MAPK. We then evaluated the mRNA expression levels of *Krt1*, *Krt10*, *Dsg1a*, and *Dsc1* after treating keratinocytes with U0126, a specific inhibitor of MEK (MAPK/extracellular signal–regulated kinase kinase) that acts upstream of p44/42 MAPK. Compared with the keratinocytes treated with vehicle in the absence of IL-4 (Figure 4b: DMSO/none), the keratinocytes treated with vehicle in the presence of IL-4 (Figure 4b: DMSO/IL-4) showed attenuated mRNA expression levels of *Krt1*, *Krt10*, and *Dsc1*. However, the attenuated mRNA expression levels of *Krt1* and *Krt10* were completely restored in the U0126-treated keratinocytes, even in the presence of IL-4 (Figure 4b: DMSO/IL-4 vs. U0126/IL-4). U0126 had no effect on the IL-4-mediated downregulation of *Dsc1* mRNA (Figure 4b: DMSO/IL-4 vs. U0126/IL-4). Taken together, these data suggest that the p44/42 MAPK signaling pathway has an essential role in IL-4-mediated changes in the mRNA expression levels of *Krt1* and *Krt10*, and possibly *Dsg1a*, but not *Dsc1*, in mouse differentiating keratinocytes.

We also examined whether the IL-4-mediated downregulation of structural components of differentiating keratinocytes depends on another MAPK, p38. The prolonged exposure of differentiating mouse keratinocytes to IL-4 or IL-13 resulted in the phosphorylation of p38 MAPK (Figure 4c). Using an inhibitor of p38 MAPK SB203580, we examined whether the p38 MAPK signaling pathway is involved in the attenuated expression of genes encoding structural components of differentiating keratinocytes. Unlike that of p44/42 MAPK, the inhibition of p38 MAPK did not restore the attenuated mRNA expression levels of *Krt1*, *Krt10*, *Dsg1*, and *Dsc1*

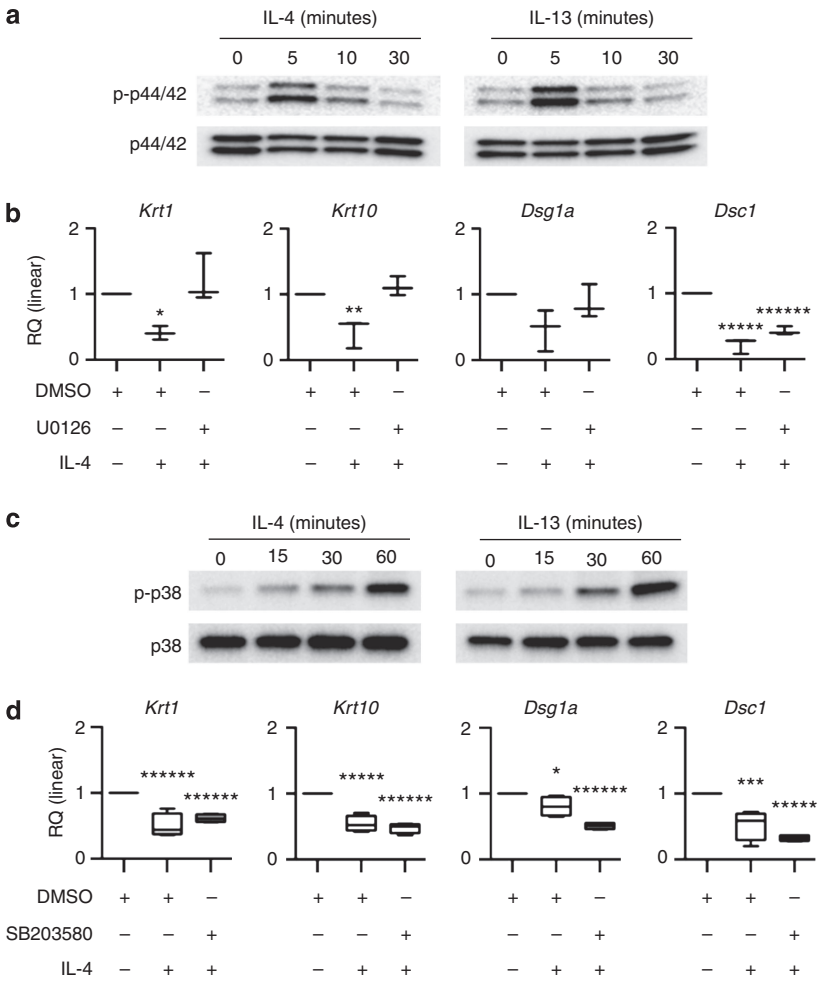

Figure 4. IL-4 downregulates the expression of genes encoding structural components of primary mouse keratinocytes via the p44/42 mitogen-activated protein kinase (MAPK) signaling pathway. A western blotting analysis of phospho-p44/42 MAPK, total p44/42 MAPK (**a**), phospho-p38 MAPK, or total-p38 MAPK (**c**) ($n = 3$). The keratinocytes were differentiated for 6 days. After overnight starvation, the cells were stimulated with IL-4 or IL-13 for 5, 10, or 30 minutes (**a**) or 15, 30, or 60 minutes (**c**). (**b**, **d**) A real-time PCR analysis of *Krt1*, *Krt10*, *Dsg1a*, and *Dsc1*. The keratinocytes were differentiated for 7 days. The cells were treated with either U0126 ($n = 3$) or SB203580 ($n = 4$) and then stimulated with IL-4 for 24 hours. Refer to Figure 1 for the normalization of the data and the relative quantities (RQs) of the genes. Asterisk, statistically significant (one-way analysis of variance with Dunnett's *post hoc* test).

induced by IL-4 (Figure 4d: DMSO/IL-4 vs. SB203580/IL-4), thus demonstrating that the p38 MAPK signaling pathway is not involved in the regulation of *Krt1*, *Krt10*, *Dsg1*, and *Dsc1* mRNA by IL-4.

The presence of IL-4 or IL-13 attenuated the mRNA expression levels of *KRT1*, *KRT10*, *DSG1*, and *DSC1* in human keratinocyte HaCaT cells and disrupted the integrity of the cell sheets

We investigated the mRNA and protein expression of differentiating human keratinocyte HaCaT cells. The genes *KRT1* and *KRT10* encoding KRTs and *DSG1* and *DSC1* encoding desmosomal cadherins in the keratinocytes were attenuated in the presence of IL-4 or IL-13, whereas the genes *KRT5*, *KRT14*, *DSG3*, and *DSC3* were expressed at similar or slightly lower levels than those observed in the control (Figure 5a and b). As observed with the mRNA expression, the protein expression levels of KRT1 and KRT10 were also attenuated, whereas the expression of DSC1 remained at a similar level to that observed in the control (Figure 5c). According to densitometry plots (Figure 5c), the expression of DSG1 differed between the IL-4 and IL-13 treatments, and the results showed that the expression of DSG1 was slightly downregulated by IL-13, but not IL-4, treatment.

We also assessed the effects of the IL-4- or IL-13-mediated downregulation of genes at early stages on the integrity of differentiating keratinocytes. Cell sheets of differentiating keratinocytes cultured in the presence or absence of IL-4 or IL-13 were dissociated, and the extent of fragmentation by mechanical stress was analyzed. As shown in Figure 5d, a larger number of fragments of cell sheets were observed four times in the cells cultured with either IL-4 or IL-13 than in the control cells. These data suggest that the presence of IL-4 or IL-13 disrupts normal processes of differentiation in keratinocytes and thus the integrity of keratinocyte cell sheets.

DISCUSSION

By using mouse and human keratinocytes *in vitro*, we herein reported that IL-4 or IL-13 attenuated the mRNA expression

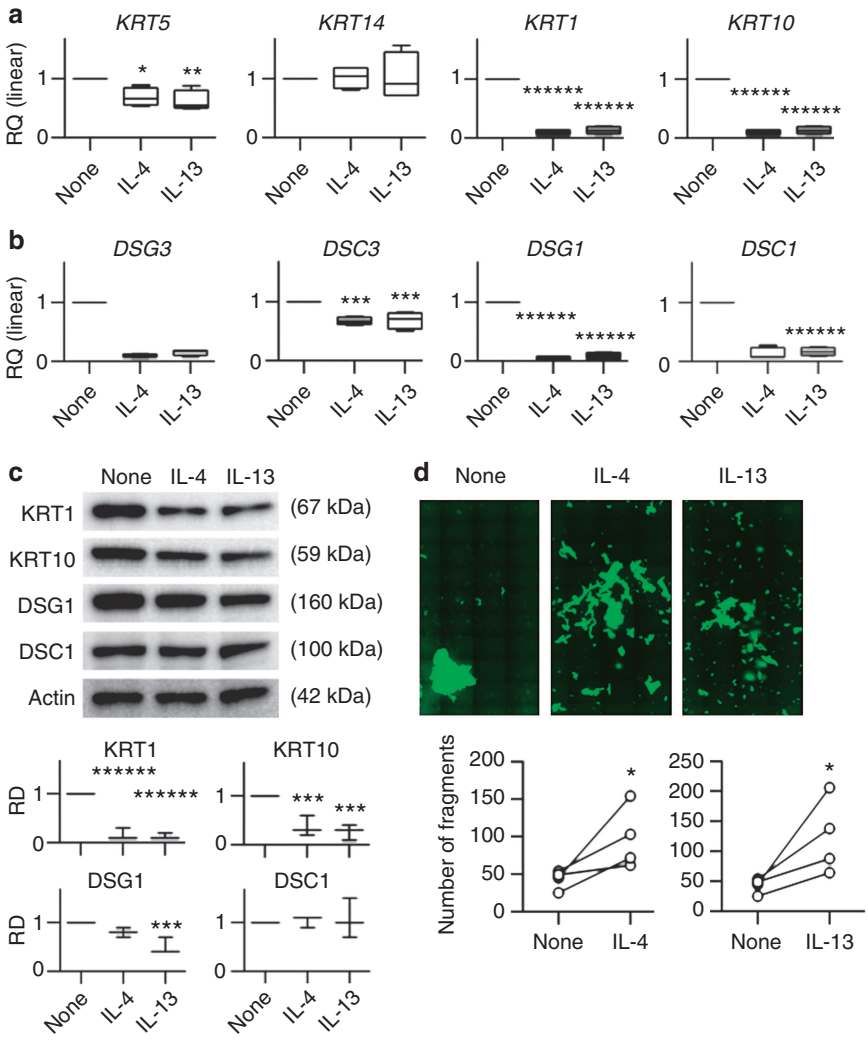

Figure 5. IL-4 or IL-13 attenuates the mRNA expression levels of *KRT1, KRT10, DSG1,* and *DSC1* and increases the susceptibility of human keratinocyte HaCaT cell sheets to mechanical stress. The keratinocytes were differentiated for 5 days. IL-4 or IL-13 was added for the last 2 days of the culture. A real-time PCR analysis of (**a**) *KRT1*, *KRT5*, *KRT10*, *KRT14*, (**b**) *DSG1*, *DSG3*, *DSC1*, and *DSC3* with or without cytokine stimulation. The data were normalized to the *TBP* mRNA expression using the comparative *CT* method. The relative quantities (RQs) of the genes ($n = 4$). Asterisks in **a** and **b**, statistically significant (one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test). (**c**) A western blotting analysis of keratin 1 (KRT1), KRT10, desmoglein 1 (DSG1), desmocollin 1 (DSC1), and actin ($n = 3$). Densitometry plots are depicted. RD, relative density; asterisk, statistically significant (one-way ANOVA with Dunnett's *post hoc* test). (**d**) The degree of keratinocyte clustering in the presence or absence of cytokines was depicted (upper) and quantified (bottom) using a dispase-based assay ($n = 4$). Asterisk, statistically significant (a matched-pair analysis).

levels of KRT1, KRT10, DSG1, and DSC1, which encode structural components of keratinocytes, at early stages. The attenuated expression was mediated by STAT6 alone or via both the STAT6 and p44/42 MAPK signaling pathways in mouse keratinocytes. As observed in various knockout mice and *in vitro* assays, the attenuation of these components is indicative of mild hyperkeratosis, acanthosis, epidermal fragility, abrogation of the cohesion of a normal stratum corneum, ulceration of the epidermis, and barrier abnormalities (Figure 6). *Krt10*-deficient mice exhibit mild hyperkeratosis and acanthosis in the epidermis (Reichelt and Magin, 2002). The deletion of both *Krt1* and *Krt10* in mice demonstrates that both genes are essential for epidermal stability (Wallace *et al.*, 2012). IL-4 reduces the cohesiveness of the stratum corneum and the expression of *DSG1* in human differentiating keratinocytes (Hatano *et al.*, 2013). Mice lacking *Dsc1* possess a fragile epidermis and develop acantholysis soon after birth, whereas adult null mice exhibit ulcerating lesions resembling chronic dermatitis (Chidgey *et al.*, 2001). A lack of *Flg* results in barrier abnormalities and thus reduces the inflammatory threshold to topical irritants and haptens (Scharschmidt *et al.*, 2009). The loss of *Lor* confers temporal instability of the stratum corneum within 5 days after birth (Koch *et al.*, 2000). The dissociation assay in the present study also showed that IL-4 or IL-13 disrupted the integrity of keratinocyte sheets (Figure 5d), although whether the fragmentation of the cell sheets resulted from the disruption of intercellular adhesion and/or the structural integrity of the cells themselves remains to be investigated (Seltmann *et al.*, 2013). Therefore, the present and previous studies suggest that IL-4 or

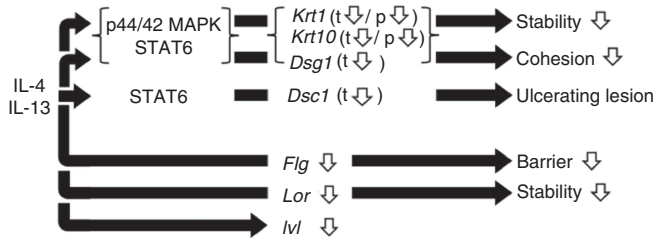

Figure 6. Schematic representation of IL-4- or IL-13-mediated changes in the structural components of differentiating keratinocytes. The presentation was summarized using previously published studies and the present study. In the presence of IL-4 or IL-13, the expression levels of genes (*Krt1*, *Krt10*, *Dsg1*, and *Dsc1*) encoding structural components of differentiating keratinocytes at early stages is downregulated via the signal transducer and activator of transcription factor 6 (STAT6) and/or p44/42 mitogen-activated protein kinase (MAPK) signaling pathways, as compared with the normal expression levels of these genes. This change may result in epidermal instability, abrogation of the cohesion of a normal stratum corneum, or ulcerating lesions, as previously reported (Chidgey *et al.*, 2001; Wallace *et al.*, 2012; Hatano *et al.*, 2013). IL-4 and/or IL-13 are capable of downregulating the expression levels of the genes *Flg*, *Lor*, and *Ivl*, which encode structural components of differentiating keratinocytes at late stages (Howell *et al.*, 2007; Kim *et al.*, 2008; Sehra *et al.*, 2010). The attenuated expression levels of *Flg* and *Lor* results in barrier abnormalities and temporal instability, respectively (Koch *et al.*, 2000; Scharschmidt *et al.*, 2009).

IL-13 attenuates the expression levels of KRT1, KRT10, DSG1, and DSC1, as well as filaggrin, loricrin, and involucrin, and disrupt the normal integrity of the keratinocyte architecture at earlier stages of mouse and human keratinocyte differentiation (Figure 6).

As keratinocytes, unlike T cells and B cells, do not express a common γ chain (γc), IL-4 and IL-13 both appear to signal through the receptor composed of the IL-4Rα and IL-13Rα1 chains (Wery-Zennaro *et al.*, 1999). In the present study, the expression levels of *Krt1*, *Krt10*, *Dsg1*, and *Dsc1* were not attenuated in the mouse *Il4ra*- or *Stat6*-deficient differentiating keratinocytes. Although the addition of either IL-4 or IL-13 induced the phosphorylation of STAT6 in the differentiating mouse keratinocytes, the expression levels of KRT1 and KRT10 were not altered upon transfection of the cells with an active form of STAT6 (Figure 3d). Therefore, our data suggest that STAT6 may require other unknown IL-4- or IL-13-mediated signals, possibly the p44/42 MAPK signaling pathway (Figure 4b), to allow the IL-4- or IL-13-mediated regulation of the *Krt1*, *Krt10*, and *Dsg1a* expression levels. Whether the expression of *Dsc1* requires the activation of STAT6 alone or a combination of STAT6 and other factors remains to be investigated (Figure 6). The p38 MAPK pathway does not appear to be involved in the IL-4-mediated attenuation of KRTs and desmosomal cadherins, whereas the activation of p38 MAPK results in blister formation via the internalization of Dsg3 and Dsc3 (Mao *et al.*, 2011). Whether the signal pathways we investigated in mouse keratinocytes are identical to those observed in human keratinocytes remains to be investigated.

In our *in vitro* study, IL-4 did not clearly downregulate the protein expression of DSG1 or DSC1, whereas IL-13 downregulated the protein expression of DSG1 only, according to the densitometry plots (Figure 5c). In fact, differentiated human keratinocyte HaCaT cells that were cultured for a long time (12 days) did not show a different expression of DSC1 between cells treated with or without IL-4 or IL-13 (Supplementary Figure S1 online). As IL-4 or IL-13 downregulated the mRNA expression levels of both genes, our results of the protein expression of both genes raise a question regarding the regulatory interplay of DSG1 and DSC1 involving the transcription, translation, and turnover of mRNA and proteins during the exposure of keratinocytes to IL-4 or IL-13. Furthermore, the attenuated expression levels of *Dsg1a* and *Dsc1* mRNA in IL-4- or IL-13-stimulated keratinocytes possibly results from the concomitant attenuation of KRT1 and KRT10 (Wallace *et al.*, 2012). On the other hand, the IL-4- or IL-13-mediated downregulated expression levels of *Krt1*, *Krt10*, and *Dsc1* mRNA may result from the concomitant attenuation of DSG1 (Harmon *et al.*, 2013). Therefore, a series of studies is needed to investigate the regulatory network of genes encoding structural components in the epidermis and the possible interplay of macromolecules during the exposure of keratinocytes to IL-4 or IL-13.

MATERIALS AND METHODS

Mice

BALB/c mice and *Il4ra*-deficient mice were purchased from Charles River Laboratories Japan (Yokohama, Japan) and Jackson Laboratory (Bar Harbor, ME), respectively. Dr S. Akira and Dr T. Nakayama kindly provided the *Stat6*-deficient mice. The animals were housed in the animal facility of the Department of Microbiology and Immunology, Tokyo Women's Medical University, Tokyo, Japan. All experiments were performed under the guidelines and protocols approved by the Animal Care, Use and Ethics Committee for animal experiments and the Safety Committee of Molecular Cloning and Use at the Tokyo Women's Medical University.

Keratinocyte cultures

The primary culture of mouse keratinocytes was performed according to the CELLnTEC protocol (described in the Supplementary Materials and methods online). The keratinocytes were differentiated in CnT-02 epithelium medium (CELLnTEC, Bern, Switzerland) supplemented with 1.2 mM $CaCl_2$ for the indicated periods.

Immortalized human keratinocyte HaCaT cells were purchased from Cosmo Bio (Tokyo, Japan). The cell line was originally established by Dr Boukamp and Dr Fusenig (Boukamp *et al.*, 1988), and Deutsches Krebsforschungszentrum (Germany) reserves all rights for its use. The HaCaT keratinocytes were grown in Dulbecco's modified Eagle's medium (Gibco, Life Technologies, Tokyo, Japan) containing 10% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37 °C and differentiated in medium containing 1.8 mM $CaCl_2$ for 5 days.

Cytokines and reagents

Either mouse recombinant IL-4 (Wako, Osaka, Japan), IL-5, or IL-13 or human IL-4 or IL-13 (Peprotech, Rocky Hill, NJ) were used for the experiments. In the experiments with an inhibitor treatment, the MEK inhibitor U0126 (Promega, Tokyo, Japan) or the p38 MAPK inhibitor SB203580 (Calbiochem, Merck Millipore, Tokyo, Japan) was used. See the Supplementary Materials and methods for more details.

Semiquantitative real-time reverse-transcriptase–PCR

cDNA was synthesized using SuperScript II (Invitrogen, Life Technologies) according to the manufacturer's instructions. Real-time PCR was performed using the comparative *CT* method: all data in mice and humans were normalized to *Ppia* (encoding cyclophilin A) and *TBP*, respectively. The data were expressed as the relative quantity of the target genes to the untreated or vehicle-treated keratinocytes. The details are described in the Supplementary Materials and Methods online.

Western blotting

A western blotting analysis was performed as described in the Supplementary Materials and methods online. The blots were probed with anti-KRT1, anti-KRT10, anti-KRT5, anti-KRT14, anti-DSG1, anti-DSC1 or anti-actin, anti-STAT6, anti-phospho-STAT6 (Thy641), anti-p44/42 MAPK, anti-phospho-p44/42 MAPK, anti-p38 MAPK, and anti-phospho-p38 MAPK (Thr180/Thr182) primary antibodies.

Transfection of keratinocytes for intracellular staining of KRTs

Keratinocytes were co-transfected with either pCMV-Myc-STAT6 or the mock plasmid plus the pEGFP-C1 vector. After transfection, the cells were used for intracellular KRT staining. The cells were stained with either anti-KRT1 or anti-KRT10 (Covance, Research Products, Denver, PA), visualized with Alexa 647–conjugated anti-rabbit Ig (Molecular Probes, Life Technologies), and analyzed with flow cytometry on an FACSCalibur flow cytometer (Becton Dickinson Japan, Tokyo, Japan). See the Supplementary Materials and Methods online for details.

Dispase-based keratinocyte dissociation assay

The dispase-based keratinocyte dissociation assay was partially modified from a previous study (Heupel *et al.*, 2009), as described in the Supplementary Materials and Methods online. Human keratinocyte HaCaT cells were labeled with CFSE (carboxyfluorescein succinimidyl ester), washed, and incubated with dispase II for 30 minutes at 37 °C. After carefully removing the dispase solution, 0.5 ml of Hank's Balanced Salt Solution was added. The cell layer was pipetted 10 times with a 1-ml pipette at a constant speed. The cells were observed under a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Statistical analysis

Differences between the two groups were tested using matched-pair analyses, whereas differences among multiple groups were tested using one-way analysis of variance with Dunnett's *post hoc* test. All analyses were performed with the JMP ver. 10.0 software program (SAS Institute, Cary, NC). The statistical significance is described in the figures (*$P<0.05$; **$P<0.01$; ***$P<0.005$; ****$P<0.001$; *****$P<0.0005$; ******$P<0.0001$).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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